Hydroalcoholic Extract of *Levisticum officinale* Increases cGMP Signaling Pathway by Down-Regulating PDE5 Expression and Induction of Apoptosis in MCF-7 and MDA-MB-468 Breast Cancer Cell Lines

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**ABSTRACT**

**Background:** This study aimed to investigate *Levisticum officinale* hydroalcoholic extract (LOHE) effect on both cGMP signaling pathway and phosphodiesterase 5 (PDE5) gene expression pattern and to examine the role of LOHE in apoptosis induction of MCF-7 and MDA-MB-468 cell lines. **Methods:** The half maximal inhibitory concentration (IC\(50\)) of LOHE was examined in both cell lines using the MTT assay. Using IC\(50\) values of LOHE on both cells, the type of cell death was detected by flowcytometric analysis. The values of PDE5 and cGMP were evaluated by real-time PCR and ELISA methods, respectively. **Results:** The IC\(50\) values were measured as 150 \(\mu\)g/ml for MDA-MB-468 and 200 \(\mu\)g/ml for MCF-7. At 12 hour of treatment, a significant decrease in the PDE5 expression and maximum increase in the amount of intracellular cGMP were observed (\(p < 0.05\)). However, these effects were more noticeable in MDA-MB-468 triple-negative cells. **Conclusion:** Our data suggest that LOHE extract could be a potential source for new strategies towards targeting both PDE5 and cGMP signaling pathways. **DOI:** 10.29252/ibj.23.4.280

**Keywords:** Apoptosis, Breast cancer, Levisticum, PDE5

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**INTRODUCTION**

Breast cancer is a type of malignancy originating from breast tissue and may spread to other vital organs such as bones, lung, liver, and brain\(^[1]\). This cancer is one of the most common malignancies in women and the second leading cause of cancer-related death among females worldwide\(^[2]\). Based on world statistics, there is an increasing incidence of breast cancer, especially in developing countries where the lower rates of this sex-related tumor have been reported\(^[3]\). Despite all efforts to prevent or treat, the disease has remained a major global health problem\(^[4]\). Exploration of novel markers for early diagnosis and therapeutic targets, which are directly involved in cancerous cell pathways, can be considered as a major challenge in cancer research\(^[5,6]\). A novel cancer-related marker is phosphodiesterase (PDE) enzyme. The PDE enzyme family members are in fact metallohydrolase proteins that break down the phosphodiester bond of the cyclic nucleotides cyclic adenosine monophosphate (cAMP) and/or cyclic guanosine monophosphate (cGMP) into the inactive 5’-AMP or GMP\(^[7,8]\). PDE5 mechanism of action is specific for hydrolysis of cGMP. PDE5 is also the predominant isoform that hydrolyzes cGMP in almost all tissues, which later causes cGMP signaling pathway to be terminated as long as intracellular cGMP levels are controlled by...
negative feedback regulation\(^{[6-10]}\). cGMP can activate the cGMP-dependent protein kinase (PKG) and PDE enzymes. It also causes ion fluxes and protein phosphorylation that can affect genes expression or other cellular responses\(^{[6,11,12]}\). Recently, the overexpression of PDE5 has been reported in several human carcinomas, including breast cancer, and suggested that PDE5 expression has positive correlation with tumor grade, lymph node involvement, and invasive potential, as well as decreasing survival rate in patients. Additionally, cGMP amount and PKG seem to have negative association with each other\(^{[13,14,15]}\). PDE5 is differentially expressed in every subtype of breast cancer cells, including luminal A (ER-positive/PR + HER2 negative), luminal B (ER + and/or PR + HER2+), HER2-enriched (ER- and PR-/HER2+), and triple-negative (i.e., the lack of all three receptors). Luminal A subtype, like the MCF-7 cell line, is the most frequent breast cancer subtype that is low grade, tends to grow slowly and has the highest survival rate, whereas triple-negative, similar to MDA-MB-468, is less common breast cancer subtype, has the lowest survival rate, has very high aggressive potentials and is difficult to treat because no hormone receptor has been found to target yet. This subtype expresses higher levels of PDE5 compared to other subtypes\(^{[16,17]}\).

Several studies have suggested that PDE5 and cGMP signaling pathways can be considered as new candidates for discovering novel therapeutic strategies toward treating breast cancer, especially triple-negative subtype\(^{[12,18,19]}\). Nevertheless, common inhibitors of PDE5 have been reported to possess a well-established side effect in clinical experiments. Extracts of plants or their bioactive compounds are found to be safe and are widely acceptable in cancer therapy\(^{[20-22]}\). Thus, herbal plants are essentially a potential source for developing novel drugs in the treatment of cancer\(^{[23-25]}\). Studies have shown that some plant extracts have anticancer activities by inhibiting proliferation and inducing cell cycle arrest beside suppressing tumor progression in vitro and in vivo\(^{[26-27]}\).

*Levisticum officinale* (lovage) is regarded as a herbaceous perennial herb of the family Umbelliferae ( Apiaceae). It is a wild herb that grows in various areas of Europe, Afghanistan, and Iran (i.e. Kerman Province). According to previous investigations, lovage is used to treat sore throats, fever, kidney stones, urethritis, congestion, rheumatism, migraine headache, and indigestion and also applied as a wound antiseptic. Lovage has also used as an appetizer, and a potent diuretic\(^{[28-30]}\) and has anti-inflammatory, antioxidant, anti-tumoral and anti-bacterial properties\(^{[28,31-33]}\). Experiments have revealed that hydroalcoholic extract of lovage (LOHE) has anti-cancer effects by induction of apoptosis in several cancer cell lines\(^{[32,34,35]}\), based on the fact that plants containing flavonoids can cause PDE inhibition. The aim of the present study was to investigate the effect of LOHE on both PDE5 expression and cGMP signaling pathway to evaluate the role of LOHE in apoptosis induction in both breast cancer cell lines, MCF-7 (ER+, PR+, HER2-), and MDA-MB-468 (triple-negative).

**MATERIALS AND METHODS**

**Chemicals and reagents**

RPMI 1640 culture medium, FBS, PBS, penicillin, streptomycin, and Trypsin/EDTA solution were all purchased from Gibco (Rockville, MD, USA). MTT, Trypan blue, and dimethyl sulfoxide (DMSO) were procured from Sigma Aldrich (St. Louis, MO, USA). The Annexin V/PI apoptosis detection kit was obtained from BioVision (San Francisco, CA, USA). The RevertAid M-MuLV Reverse Transcriptase and the cGMP Direct Immunoassay kit were obtained from Takara Bio Inc. (Dalian, China) and R&D Systems (Minneapolis, MN, USA), respectively. All other materials were of analytical grade.

**Plant materials**

*L. officinale* (lovage) was collected in spring time from a small area in Southeast of Iran, Hazar Mountains, ranging from 3000 to 3400 meters from the sea level. Taxonomy of Lovage was confirmed by Department of Biology, University of Sistan and Baluchistan, Zahedan, Iran.

**Preparation of hydroalcoholic extract**

Extract of 70% water-alcohol was prepared with the Soxhlet extractor. The aerial part of the plant was dried in the dark at room temperature. Then 20 g of the dried plant stems and leaves were added to 300 ml of alcohol 70% and placed in the Soxhlet device. Afterwards, the extract was filtered (Whatman No. 41), and the solvent was removed using a freeze dryer machine (MAXI DRY-LOY, Heto-Holten, Allerod, Denmark). Primary Stock made from 100 mg of LOHE was dissolved in 1 ml of DMSO (HPLC grade) and kept in -20 °C for further use.

**Cell culturing**

Human breast cancer cell lines, MCF-7 and MDA-MB-468, were purchased from the National Cell Bank of Pasteur Institute of Iran (Tehran). The cells were

cultivated in RPMI 1640 medium containing 10% FBS, 100 U/ml of penicillin, and 100 μg/mL of streptomycin under standard cell culture conditions (95% humidity, 37 °C, 5% CO₂). The culture medium within the flask was replaced with a fresh medium every 2-3 days. Prior to each assay, cells were counted, and the number of living cells were calculated with Hemocytometer and by Trypan blue assay. All assays were done at least in triplicates.

Cell viability
MTT assay was used to evaluate cytotoxicity. Almost 5000 cells per well were seeded in 96-well microplates and let to grow until the confluency of 80%. The culture medium was removed, and cells were treated with the concentrations of 0 (DMSO containing culture medium), 50, 100, 150, 200, 300, 400, and 500 μg/mL of LOHE and incubated for 24, 48, and 72 hours. Then 20 μL of MTT solution was added to treated and untreated cells. Following 4 h of incubation at 37 °C, the solution within microwells was carefully drained, and 150 μL of DMSO was added to each microwell and kept in the dark for 20 min. Absorptions were read at 570 nm using a microplate reader (Stat Fax 2100; Awareness Technology, Los Angeles, CA, USA), and cell viability was measured.

Apoptosis assay
Apoptotic induction rate in cells treated with different concentrations of the extract, and untreated cells was evaluated using Annexin V-FITC apoptosis detection kit (BioVision) according to the manufacturer’s protocol. Briefly, 1 × 10⁵ cells per well were seeded in six-well plates. Then the cells were treated with increasing doses of LOHE, 0, 50, 100, 150, 200 μg/ml for MDA-Mb-468 and 0, 100, 200, 300 μg/ml for MCF-7. Afterwards, the medium was discarded, and the cell pellet was transferred to a microtube before centrifuging at 12000 rpm for 10 minutes. Later, 500 μL of 1× binding buffer and 5 μL of Annexin V-FITC were added to cell suspension and incubated in the darkness at room temperature for 10 minutes. Finally, 5 μL of propidium iodide was added to cell pellets, and the samples were analyzed by using a Pas-II cytometer (Partec AG, CH-4144 Arlesheim, Switzerland).

Extraction of total RNA and synthesis of complementary DNA (cDNA)
Cells were cultured in six plates (1 × 10⁵ cells per well) and were treated with the concentration equal to IC50 values of LOHE for both cells (150 μg/ml for MDA-MB-468 and 200 μg/ml for MCF-7) at 2, 4, 8, 12, and 24 h after treatment. Total RNA was isolated using RNX (SinaClon, Tehran, Iran) according to the manufacturer’s instruction. cDNA was synthesized using the Takara first strand cDNA synthesis kit (Dalian, China) based on the protocol provided by manufacturer.

Real-time polymerase chain reaction (PCR) assay
Real-time PCR was performed for relative mRNA expression of PDE5. The PDE5 primers were designed, ensuring that all the isoforms of PDE5 were amplified. PDE5 and GAPDH primer sequences were as follows: 5'-TGTTGGTGTAGCACAGACCA-3' and 5'-GAGCCACATCGCTCAGAC-3' as forward and 5'-GCAGTGAAAGTCTGATAGAGC and 5'-CATGTAGTTGAGGTCATGAAGG-3' as reverse primers, respectively. PCR amplification consisted of 35 cycles: 95 °C for 15 seconds, 58.5 °C for 30 seconds, and 72 °C for 45 seconds. The housekeeping gene, GAPDH, was used to normalize the relative expression level of the PDE5 gene. Relative expression of PDE5 was compared with GAPDH (as the internal control) using the comparative 2−ΔΔct method.

Measurement of cGMP concentration
cGMP levels in both cells were measured by ELISA method using the cGMP direct immunoassay kit (R&D Systems, Minneapolis, MN, USA). Briefly, in this assay, cGMP present in a sample competes with a constant amount of peroxidase-conjugated cGMP for sites on rabbit polyclonal antibody during the incubation. Hence, the polyclonal antibody binds the pre-coated goat anti-rabbit antibody. Eventually, cGMP levels were measured based on absorptions at 450 nm.

Statistical analysis
SPSS software version 22 (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis of the data, and the findings were reported as mean ± SD. The statistical analysis was conducted by a nonparametric analysis of variance between the groups. p < 0.05 was considered statistically significant in all assays.

RESULTS

Antiproliferative effects of LOHE
LOHE significantly reduced viability in both MCF-7 (Fig. 1A) and MDA-MB-468 (Fig. 1B) cell lines in a concentration- and time-dependent manner (p < 0.05). The most significant inhibitory effect of LOHE was at 300 μg/ml and 500 μg/ml after 72 h of treatment in MDA-MB-468 and MCF-7 cell lines, respectively. Moreover, the results showed that the IC50 values of
**LOHE** were 150 μg/ml for MDA-MB-468 cells and 200 μg/ml for MCF-7, following 48 h of treatment.

**Induction of apoptosis by LOHE**

To investigate the apoptosis inducing potency of **LOHE**, MCF-7 and MDA-MB-468 cell lines were treated with different concentrations of this extract and incubated for 48 h. As shown in Figure 2, there were significant increases in the early and late apoptosis rate of both cells in a concentration-dependent manner ($p < 0.05$), but the fraction of MDA-MB-468 cells undergoing apoptosis was higher compared to MCF-7 cells treated with the same concentrations of **LOHE**.

**Effect of LOHE on PDE5 mRNA gene expression**

Effect of **LOHE** on **PDE5** expression was assessed in a time-dependent manner in both MCF-7 and MDA-MB-468 cell lines. As shown in Figure 3, the **PDE5** mRNA levels significantly reduced in MCF-7 cells in the presence of **LOHE** over treatment periods of 2, 4, 8, 12, and 24 h compared to that of the untreated groups ($p < 0.05$). A similar effect was found in MDA-MB-468 cells as well. However, the inhibitory effect of **LOHE** on MDA-MB-468 was more significant than MCF-7 cells in all the measured periods ($p < 0.05$).

**cGMP intracellular assay**

As shown in Figure 4, **LOHE** significantly increased cGMP concentrations at 8-12 h exposure periods in both MCF-7 and MDA-MB-468 cell lines compared to untreated cell lines ($p < 0.05$). The highest cGMP levels in MCF-7 and MDA-MB-468 cell lines were at 12 h treatment. Results indicated that intracellular cGMP levels increased in both cell lines following treatment with **LOHE** compared to the adjacent untreated controls ($p < 0.05$). As a result, promoted level of cGMP suggests the presence of a major regulator of basal cGMP levels in both cell lines.

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**Fig. 1.** Concentration-response analysis of **LOHE** on both (A) MCF-7 and (B) MDA-MB-468 cells ($p < 0.05$).

**Fig. 2.** Flowcytometric analysis of death cell types after 48 h exposure to different concentrations of **LOHE** in (A) MCF-7 and (B) MDA-MB-468 cell lines ($p < 0.05$).
Dysregulation of Wnt signaling plays a crucial role in the development and progression of triple-negative breast cancer. It has been reported that flavonoids, especially quercetin and coumarin, are able to inhibit potential Wnt/β-catenin signaling.

Regarding the effects of LOHE on PDE5 mRNA levels, there were significant differences between MDA-MB-468 and MCF-7 cells at the times of 2-24 h, particularly at 12-h in this period. The mRNA level increased after 24 h in both cell lines, even though the elevation in PDE5 mRNA in MDA-MB-468 cell line was more evident than that of MCF-7 cell line. Our results showed that LOHE could increase significantly the intracellular cGMP levels in both cell lines. This feature of LOHE was correlated with adjacent PDE5 expression alterations and intracellular cGMP levels. As an example, during 8-12 h, PDE5 expression was minimum, and consequently, cGMP levels were maximum. However, the increased amounts of cGMP showed anti-proliferative effects of LOHE in both cell lines. This increase was observed more in triple-negative MDA-MB-468 cells compared to MCF-7 cells.
In summary, our findings show that LOHE inhibits proliferation and induces apoptosis in two cell lines. In addition, LOHE could be a novel source of drug candidates in breast cancer treatment, especially for those types of cancer, which there are fewer treatment options and limited markers for pharmaceutical target (no hormone receptor). The high expression of PDE5 can be a new marker for this subtype, and inhibition of this enzyme with compounds derived from this plant may be pursued in clinic in near future.

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CONFLICT OF INTEREST. None declared.

REFERENCES

22. Balhara YPS, Sarkar S, Gupta R. Phosphodiesterase-5 inhibitors for erectile dysfunction in patients with diabetes mellitus: A systematic review and meta-


42. Lee K, Piazza AG. The interaction between the Wnt/β-catenin signaling cascade and PKG activation in cancer. *Journal of biomedical research* 2017; 31(3): 189-196.


